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Lysosomal Enzyme Precursors in Coated Vesicles Derived from the Exocytic and Endocytic Pathways

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Abstract. The molecular forms of two lysosomal enzymes, cathepsin C and cathepsin D, have been examined in lysosomes and coated vesicles (CVs) of rat liver. In addition, the relative proportion of these lysosomal enzymes residing in functionally distinct CV subpopulations was quantitated. CVs contained newly synthesized precursor forms of the enzymes in contrast to lysosomes where only the mature forms were detected. Exocytic and endocytic CV subpopulations were prepared by two completely different protocols. One procedure, a density shift method, uses cholinesterase to alter the density of CVs derived from exocytic or endocytic pathways. The other relies on electrophoretic heterogeneity to accomplish the CV subfractionation. Subpopulations of CVs prepared by either procedure showed similar results, when examined for their relative proportion of cathepsin C and cathepsin D precursors. Within the starting CV preparation, exocytic CVs contained approximately 80–90% of the total steady-state levels of these enzymes while the level in the endocytic population was ~10–13%. The implications of these findings are discussed with regard to lysosome trafficking.

THE transport of lysosomal enzymes from their site of synthesis in the rough endoplasmic reticulum to the lysosome is a complex, multistep process. During transport, the enzymes pass through numerous cellular compartments and along the way to the lysosome they are modified by the addition of carbohydrate side chains containing mannose 6-phosphate (M6P)[1] moieties which serve as recognition markers for the binding of the enzymes to specific membrane receptors (M6P receptors). These receptors participate in enzyme sorting and they are thought to play a role in enzyme transport as well. The structure of the recognition markers, and the biosynthetic reactions which lead to their formation, are understood in considerable detail. In addition, much is known about the structure and the physical characteristics of the specific lysosomal enzyme receptors (for reviews see references 18 and 30). Little is known, however, about the details of enzyme sorting and transport.

Considerable evidence indicates that clathrin-coated vesicles (CVs) are involved in the process of protein transport (for review see references 7, 8, and 13). Furthermore, early morphological studies suggest that these vesicles are also involved in lysosomal enzyme transport (10, 15, 24).

CVs isolated from rat liver, calf brain, and human placenta have been shown to contain newly synthesized lysosomal enzymes (5, 29) bound to M6P receptors (4, 28). Localization at the EM level has shown that in rat liver, the coated vesicles containing M6P receptors appear to be concentrated in the *trans*-Golgi region (11), however, they are also detected in the area of the *cis*-Golgi (2). Recent studies suggest that coated vesicles shuttle M6P receptors between the Golgi and a discrete population of endosomes (3). Coated vesicles derived from the plasma membrane by receptor-mediated endocytosis also contain M6P receptors (33, 34).

Two different procedures have been devised that allow fractionation of rat liver coated vesicles into functionally distinct subpopulations. One procedure uses a cholinesterase-mediated density shift technique to alter the density of coated vesicles derived from exocytic or endocytic pathways (14). The other uses agarose gel electrophoresis to separate exocytic and endocytic coated vesicles by their apparent differences in surface charge (17). Because lysosomal enzymes are synthesized at extremely low levels in the cell it would be difficult to use biosynthetic pulse-labeling experiments for detection of newly synthesized lysosomal enzymes in isolated subpopulations of CVs. However, the availability of specific antibodies directed against two rat liver lysosomal

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1. *Abbreviations used in this paper*: CV, clathrin-coated vesicle; M6P, mannose 6-phosphate.

enzymes has allowed us to examine the relative proportions and the molecular forms of these two lysosomal enzymes in steady-state populations of exocytic and endocytic CVs.

Materials and Methods

The iodination reagent Iodogen was purchased from Pierce Chemical Co. (Rockford, IL) and carrier free $Na^{125}I$ was from Amersham Corp. (Arlington Heights, IL). PD-10 columns were from Pharmacia Fine Chemicals (Piscataway, NJ). Immunoprecipitin was from Bethesda Research Laboratories (Bethesda, MD). The Ig fraction of a rabbit antiserum against rat liver cathepsin C (21) was provided by Dr. F. Mainferme, University of Namur. The rabbit antiserum and the affinity-purified antibodies against rat liver cathepsin D were provided by Dr. F. Baccino, University of Torino.

Coated Vesicle and Lysosome Purification

Coated vesicles were isolated by a modification (14, 17) of the method of Blitz et al. (1). As previously demonstrated (26), the CVs were at least 95% pure by morphological criteria using both thin section and negative stain EM. Over 95% of the cholinesterase activity was demonstrated to be associated with CVs (14). Coated vesicles were then subjected to the cholinesterase density shift protocol as described (14) or fractionated into subpopulations by preparative agarose gel electrophoresis which purifies CVs to apparent homogeneity (16, 17, 27). Rat liver lysosomes were prepared by applying a light mitochondrial fraction (6) to a discontinuous metrizamide gradient (32).

Iodination of Vesicles

Purified coated vesicles and lysosomes were solubilized in 50 mM Tris, pH 7.4, 0.15 M NaCl, 4.0 M urea, 1% CHAPS, and labeled for 15 min at room temperature using the Iodogen procedure (22). Free iodide was removed on PD-10 columns equilibrated with 50 mM Tris, pH 7.4, 0.15 M NaCl, 0.5% Nonidet P-40, 0.05% SDS. Specific activities were typically between 1 and 4×10^6 cpm/μg.

For iodination of subpopulations of CVs, equal amount of proteins were used. The specific activities of the various subpopulations differed by less than ±15%.

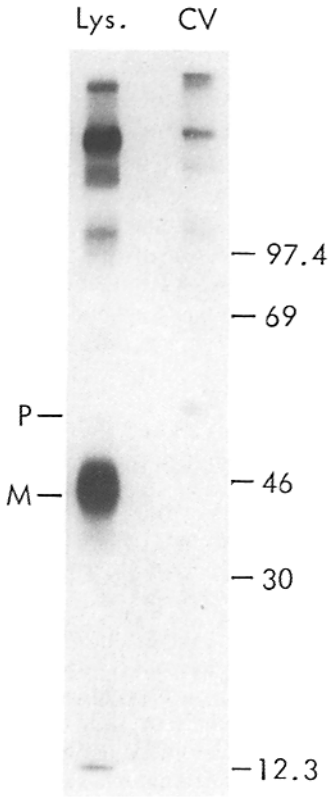


Figure 1. Molecular forms of cathepsin D in lysosomes and coated vesicles. Lysosomes (Lys) and coated vesicles (CV) isolated from rat liver were solubilized, iodinated, and subjected to immunoprecipitation with cathepsin D antiserum. The precursor (*P*) and mature (*M*) forms of rat cathepsin D are indicated. The following ^{14}C-methylated protein standards were used: phosphorylase B, 97,400; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and cytochrome C, 12,300.

Isolation and Analysis of Immunoprecipitates

Iodinated samples were adjusted to 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetamide, and 0.5% Triton X-100, and were preabsorbed by incubating at 4°C with 20 mg of Immunoprecipitin overnight and then with 10 mg of Immunoprecipitin for 30 min. The samples were subjected to centrifugation at 45,000 *g* for 1 h. Cathepsin C was immunoprecipitated from the supernatants as described (21). In some experiments the specificity of the immunoprecipitation was examined by adding an excess of purified rat liver cathepsin D or replacing anti-cathepsin C Ig with preimmune Ig. After immunoprecipitation of cathepsin C, the samples were incubated for 1–4 h with 10 mg Immunoprecipitin and subjected to centrifugation as above. Cathepsin D was immunoprecipitated from the supernatants with either antiserum or affinity-purified antibodies using the same conditions as for cathepsin C (21).

Metabolic labeling of Morris hepatoma 7777 cells with [^{35}S]methionine and immunoprecipitation of cathepsin D from extracts of cells and medium was carried out as described for cathepsin C in reference 21. The cathepsin C immunoprecipitates were solubilized in the absence of dithiothreitol (DTT) and analyzed in 15% gels, while the cathepsin D immunoprecipitates were solubilized in the presence of 10 mM DTT and analyzed in 12.5% gels by SDS PAGE (19) followed by fluorography (20). Radioactivity in polypeptides was quantified by densitometry of fluorograms.

Other Procedures

Protein was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA); all samples were incubated in 50 mM NaOH for 30 min before assay in order to improve the detection of membrane proteins.

Results

Molecular Forms of Cathepsin D and Cathepsin C in Lysosomes and CVs in Rat Liver

CVs and lysosomes were isolated from rat liver by previously described methods (17, 26, 32), and examined for their content of two lysosomal enzymes, cathepsin D and cathepsin C. The highly purified lysosomes and CVs were solubilized and iodinated and the two lysosomal enzymes were examined by immunoprecipitation and SDS PAGE. Fig. 1 illustrates the molecular forms of cathepsin D that we observed in these two organelles. Cathepsin D in lysosomes has an apparent molecular mass of 44 kD which represents the mature form of the enzyme in rat liver. In contrast, CVs contain a 53-kD form of the enzyme. Pulse-chase experiments in Morris hepatoma 7777 cells (a rat hepatoma line) identified the 53-kD form as the precursor of the mature 44-kD polypeptide (Fig. 2). Even after prolonged chase periods, the single chain 44-kD cathepsin D is not processed to a two-chain form as is typically observed in human tissues (28). From a longer exposure of the x-ray film than shown in Fig. 1 it was estimated that >85% of the cathepsin D in CV immunoprecipitates is in the precursor form. Thus, the CV preparation appears to be insignificantly contaminated with lysosomes. Although one would expect to find considerably less cathepsin D in CVs than lysosomes, the amounts seen in Fig. 1 cannot be compared quantitatively, as they are influenced by the amount of protein used in the iodinations, the relative susceptibility of the two forms to iodination, and the relative proportion of the two populations in the cell.

When the molecular forms of cathepsin C were probed by immunoprecipitation using the same CV and lysosome preparations used for cathepsin D, a complex pattern of precursor and mature polypeptides was obtained. The lysosomal form of cathepsin C consists of two groups of polypeptides, one which ranges in size from 18 to 23 kD and the other from

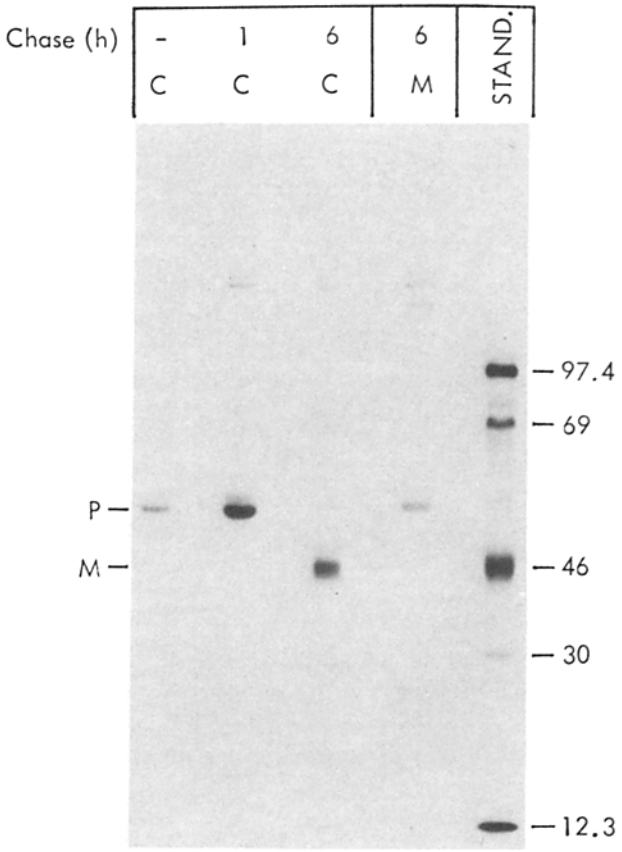


Figure 2. Molecular forms of cathepsin D in rat hepatoma (Morris hepatoma 7777) cells. Morris hepatoma 7777 cells were labeled for 10 min with [^{35}S]methionine and harvested after a chase for up to 6 h. Cathepsin D was immunoprecipitated from extracts of cells (*C*) and medium (*M*). The precursor (*P*) and mature (*M*) forms of cathepsin D are indicated. For standards see Fig. 1.

45 to 78 kD (Fig. 3, lane *2*). The coated vesicle forms of cathepsin C (Fig. 3, lane *4*) were represented by a group of polypeptides ranging from 47 to 100 kD with 57- and 67-kD polypeptides representing ~80% of the total CV-associated cathepsin C forms (see also Figs. 4 and 6). We used fivefold more CV radioactive polypeptides for immunoprecipitation in Fig. 3 (lane *4*) than in Fig. 1 (lane *2*) in order to detect even the minor CV-associated forms. Addition of excess unlabeled pure rat liver cathepsin C prevented the precipitation of all the labeled polypeptides in both the lysosome and CV preparations (results not shown).

In a previous study the precursor forms of cathepsin C were characterized in the Morris hepatoma 7777 cells (21). In these cells, the major cathepsin C precursors were two polypeptides of 59 and 63 kD, which were processed to numerous mature chains ranging from 18.5 to 61 kD. Thus, it appears that as seen with cathepsin D, the lysosomal and CV polypeptides represent mature and precursor chains of cathepsin C, respectively.

Molecular Forms of Cathepsin D and C in Subpopulations of Coated Vesicles Fractionated by Agarose Gel Electrophoresis

CVs can be fractionated on agarose gels into functionally distinct subpopulations (17). CVs which migrate fastest are the most abundant and enriched in secretory cholinesterase, while those migrating more slowly contain newly internalized asialoorosomucoid and thus are enriched in endocytic CVs. Aliquots or samples representing differently migrating subpopulations were iodinated and immunoprecipitated to determine the relative abundance of cathepsin C and cathepsin D. The molecular forms of the cathepsin C and cathepsin D polypeptides in the fastest migrating fraction (Fig. 4, lane *1*), intermediate migrating fractions (Fig. 4, lanes *2* and *3*), and slowest migrating fraction (Fig. 4, lane *4*) were all identical and contained the precursor forms of these enzymes.

The relative abundance of cathepsin C and D precursors in the various CV subpopulations was determined by densitometry of the fluorograms seen in Fig. 4. The distribution in the different fractions was calculated taking into account the relative abundance of CVs in each subfraction (Fig. 5). Cathepsin C and D precursors are for the most part associated with the fast and intermediate migrating subpopulations, while the slowest migrating CVs contain <10% of the total CV-associated precursor.

Molecular Forms of Cathepsin C in Endocytic and Exocytic Subpopulations of Coated Vesicles

Coated vesicles from the exocytic pathway can be selectively separated from the remaining CVs by a density shift procedure which depends on the presence of secretory cholinesterase (14). By using a protocol involving the inhibition of secretory cholinesterase and loading of the endocytic pathway with exogenously added cholinesterase, the endocytic CVs can be likewise separated using the same density shift manipulation. CVs containing neither secretory cholinesterase nor exogenously supplied cholinesterase (taken up by endocytosis) would represent a population of CVs that is not shifted by either procedure. CVs shifted due to their content of secretory cholinesterase (exo-shifted) and CVs shifted through exogenously supplied cholinesterase (endo-shifted) were radiolabeled and examined for their content of cathep-

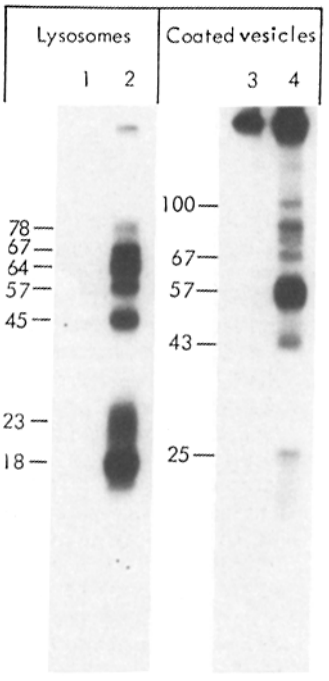


Figure 3. Molecular forms of cathepsin C in lysosomes and coated vesicles. Lysosomes and coated vesicles isolated from rat liver (see Fig. 1) were reacted with either preimmune Ig (lanes *1* and *3*) or anti-cathepsin C Ig (lanes *2* and *4*). The amount of coated vesicle protein used for immunoprecipitation was fivefold higher than that of lysosomes. The molecular weights of the cathepsin C forms are indicated.

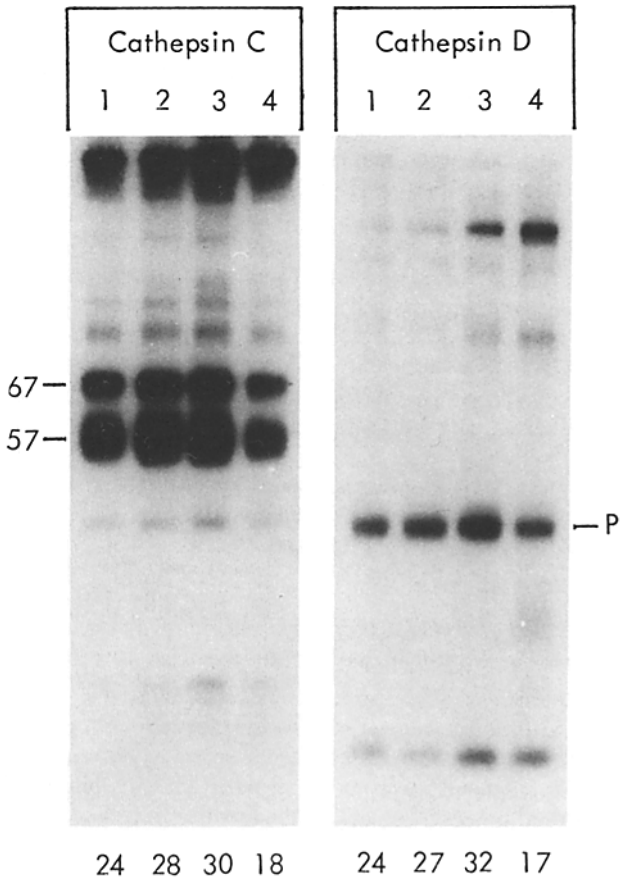


Figure 4. Cathepsin C and D in subpopulations of coated vesicles fractionated by agarose gel electrophoresis. CVs were fractionated into subpopulations 1–4 of decreasing mobility in agarose gel electrophoresis (fraction *1* representing the fastest migrating CV subpopulation). Cathepsin C (*left*) and D (*right*) were isolated by immunoprecipitation. The major M_r 57,000 and 67,000 precursors of cathepsin C and the M_r 53,000 precursor of cathepsin D are indicated. The number below each lane is the relative amount of radioactivity in cathepsin C and D in each CV subpopulation.

sin C precursors. The unshifted CVs from each experiment were likewise examined (Fig. 6). The exocytic shifted CVs contained ~90% of the total CV-associated cathepsin C precursors while the endocytic shifted CVs contained ~13% of the precursors.

Discussion

This study clearly demonstrates that rat liver coated vesicles are involved in the transport of newly synthesized lysosomal enzymes (e.g., higher molecular weight precursors). This confirms earlier studies in human skin fibroblasts where a transient association of cathepsin D precursors with CVs was found (28). Our initial studies demonstrated the presence of lysosomal enzyme precursors and their processed forms in coated vesicles from both rat liver and human placenta (5, 29). It seems likely from the present results that only precursor forms are present in CVs and that a limited proteolysis of lysosomal enzyme precursors was detected in these previous studies. The development of procedures that enable the separation of coated vesicles derived from the exocytic and endocytic pathways has allowed us to examine functionally distinct CV subfractions for their participation in lysosomal enzyme transport. The majority of CV-associated cathepsin C (~90% of the total) was recovered in the population of exocytic CVs defined by their content of endogenous cholinesterase (Fig. 6, endo shifted CVs). This would imply that the remaining 10% of the cathepsin C precursors reside in endocytic coated vesicles and/or coated vesicles that belong to neither group. By using a modified density shift protocol that allows the isolation of coated vesicles derived from the endocytic pathway we could demonstrate that ~13% of the CV-associated cathepsin C precursors reside in the endocytic pathway. It has been previously demonstrated that ~10% of the total rat liver CVs appear in the shifted region independently of endogenous or exogenous cholinesterase. This represents one-third and one-sixth of the total CVs obtained as exocytic shifted and endocytic shifted populations, respectively. Thus, the values calculated for the distribution of cathepsin C precursors in exocytic shifted (90%) and endocytic shifted (13%) coated vesicles may, in fact, be somewhat lower.

Cathepsin C in coated vesicles fractionated by agarose gel electrophoresis had a similar distribution between fractions enriched in exocytic and endocytic CVs. An inherent limitation in all studies using purified organelles is imposed by the degree of purity obtainable. Agarose gel-purified coated vesicles are exceptionally free of smooth vesicles (17), a possible source of contaminating precursor cathepsins. In the agarose gel-purified CV subpopulations, the bulk of the cathepsin C and D were associated with the fast and moderate (exocytic) CVs, which coincidentally exhibited the least amount of smooth vesicle contamination (<2%). The slower (endocytic) CVs, although contaminated to a greater degree with smooth vesicles (up to 5%), only contained minor amounts of cathepsin C (10%) and D (9%). It therefore seems very unlikely that the minor fraction of contaminating smooth vesicles contributed significantly to the amounts of cathepsin C and D which were measured in the coated vesicle subpopulations.

Thus, two completely independent procedures for subfractionation of CVs can be used to demonstrate that the majority of the lysosomal enzyme precursors present in CVs are associated with vesicles involved in the exocytic pathway.

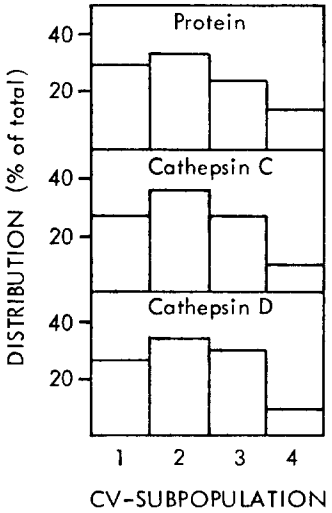
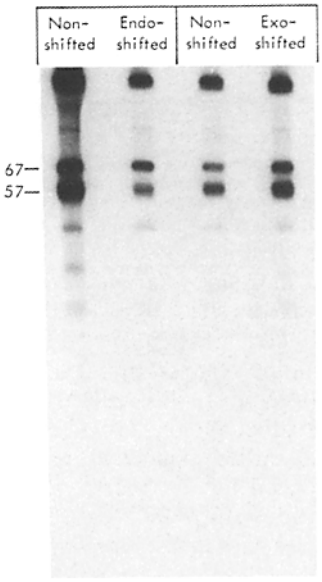


Figure 5. Distribution of cathepsin C and D in subpopulations of coated vesicles fractionated by agarose gel electrophoresis. The distribution of protein, cathepsin C, and cathepsin D in the CV subpopulations shown in Fig. 4 is given. The distribution of cathepsin C and D was calculated from the relative amount of both enzymes in the immunoprecipitates shown in Fig. 4 and the relative amount of protein in the CV subpopulations.



A	40	60	74	26
B	91	9	23	77
C	87	13	10	90

Figure 6. Cathepsin C in endocytic and exocytic subpopulations of coated vesicles. Endocytic CVs were separated by a density shift from remaining CVs (*left*). Likewise, exocytic CVs were separated by a density shift from remaining CVs (*right*). Cathepsin C was immunoprecipitated from the iodinated CV subpopulations. The major M_r 57,000 and 67,000 precursors of cathepsin C are indicated. The values below the lanes give the distribution of protein (*A*), the relative amount of radioactivity in immunoprecipitated cathepsin C precursors (*B*), and the relative amount of cathepsin C precursor (*C*, calculated from the values in *A* and *B*) in the CV subpopulations.

Along the secretory pathway, clathrin-coated membranes are enriched at the *trans* face of the Golgi and the *trans*-Golgi reticulum (25) while proximal areas of the secretory pathway are relatively poor in clathrin-coated membranes. We assume, therefore, that the majority of the cathepsin C present in exocytic CVs is likely to have already passed through the *trans* face of the Golgi. This is supported by the observation that secretory albumin, cathepsin D, and the 215-kD M6P receptor are localized in coated areas of the *trans*-Golgi reticulum of Hep G2 cells (12). If the cathepsin C and D precursors that we have detected in exocytic CVs are to end up in lysosomes, their sorting must occur at a location distal to the *trans*-Golgi reticulum. Finally, recent experiments have demonstrated that exocytic CVs, as defined by the cholinesterase-mediated density shift protocol, contain internalized asialotransferrin which has been resialylated (9). This indicates that the site of origin of these CVs is at, or distal to, the site of resialylation, i.e., the *trans*-Golgi reticulum.

At maximum, 13% of the total cathepsin C precursors were detected in endocytic CVs (Fig. 6). This lower level of precursors in endocytic CVs versus exocytic CVs can be interpreted in several ways. The bulk of the lysosomal enzymes present in the exocytic CVs may be transferred to lysosomes independently of endocytic CVs, implying that the enzyme precursors leave the secretory route before fusion with plasma membrane. Alternatively, coated vesicles derived from the endocytic pathway may have a much shorter half-life than those derived from the exocytic pathway. Were this true, the data would not contradict models whereby the bulk of the lysosomal enzyme precursors reached the plasma membrane before their delivery to lysosomes (31). Finally, it is possible that most of the lysosomal enzyme precursors present in exocytic CVs are not retained within the cells but are instead secreted (23).

It is not known whether coated vesicles constitute an obligatory transport intermediate in lysosomal enzyme packaging. Therefore, our considerations about the role of endocytic and exocytic CVs in transport of newly synthesized lysosomal enzymes apply only to that fraction of precursors which, in the steady state, are associated with CVs. If all newly synthesized lysosomal enzymes are transported within CVs and if, as discussed above, exocytic and endocytic CVs have similar half-lives in the cell, then our data would support a model whereby the bulk of newly synthesized lysosomal enzymes are transported in exocytic CVs from the Golgi to a prelysosome sorting compartment before enzyme packaging into lysosomes. Approximately 10 to 15% of newly synthesized enzymes, secreted before they reach the lysosome, can be endocytosed and transported to a prelysosomal compartment in endocytic CVs.

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